



Comparison of photoinactivation of *T. rubrum* by new methylene blue (NMB) and indocyanine green (EmunDo®)



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ABSTRACT

Background: Superficial mycotic skin infections which are predominantly caused by *Trichophyton rubrum*, poorly respond to conventional therapies. A great amount of attention has focused on finding more effective treatments. The current work is aimed to compare the effectiveness of photoinactivation of *Trichophyton rubrum* by two relatively new photosensitizers: a phenothiazinium dye (New methylene blue) and Indocyanine green (EmunDo®).

Materials and methods: A Final inoculum of *T. rubrum* which corresponded to 10⁶ colony forming unit per milliliter (CFU ml⁻¹) was prepared. Antimicrobial Photodynamic treatment (aPDT) of *T. rubrum* was carried out by either EmunDo® (1 mg/ml, Infra-red laser (IRL, λ = 810 nm, Energy Density 55 J/cm²)) or NMB (10 μM, Red laser (RL), λ = 630 nm, Energy Density of 5 J/cm²). The suspensions thereafter were subcultured on Sabouraud dextrose agar (SDA) and were counted on due time, based on colony-forming unit per milliliter (CFU/ml).

Results: aPDT with either EmunDo® (E) or NMB (N) considerably diminished the viability of inoculated *T. rubrum* with respective reduction of 0.64 log and 0.4 log compared to the control group (P < 0.001). No significant difference was found between two laser only groups (P = 0.79) and two aPDT groups (P = 0.73), however significant reduction of *T. rubrum* in red laser only group (P = 0.04) and EmunDo® only group (P = 0.04) was found as compared to the control group (P < 0.05).

Conclusion: The study provides evidence regarding satisfactory photodynamic inactivation of *T. rubrum* with EmunDo® or NMB as photosensitizers. Irradiation by only red laser source was found superior to only infra-red laser source. Dark toxicity of EmunDo® was more successful than new methylene blue dye.

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1. Introduction

Superficial mycotic skin infection (i.e., dermatophytosis), is one of the most prevalent human infections [1]. Among dermatophytes, *Trichophyton rubrum* (*T. rubrum*) is predominantly isolated from skin lesions and is one of the most common onychomycosis (fungal nail infection) [1,2]. *T. rubrum* invades skin and nail through keratin layer and the stratum corneum and causes athlete's foot in which

the skin becomes itchy and sore and may transmit from one person to the another [3].

Incidence of superficial and cutaneous fungal infections is 20–25% of world population which reveals the importance of these diseases [4]. The infection caused by this agent is not life threatening; however it is chronic and recurrent and responds poorly to treatment. Its Ability to cause chronic infection is attributed to immunosuppressive agents in cell wall components. Furthermore Pathogen's ability to produce proteolytic enzymes is the major determinant of virulence. Majority of research on dermatophytosis are focused on this microorganism [3,4]

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Treatment of dermatophytosis is based on administration of antifungal drugs which can be applied systemically or locally [5,6]. Numerous reports have been published about emergence of resistant forms of this microorganism especially in immunocompromised patients which complicated treatment process. Treatment failure and drug resistance are associated with production of resistant spores by dermatophytes. [4–6]. On the other hand, long lasting consumption of systemic antifungal drugs can cause other serious systemic side effects. Interaction with liver functions, blood dyscrasia has been frequently reported [5,6]. Hence, great attention has been focused on to find alternative therapies to improve the efficacy of treatment. A light source with specific wavelength and a photosensitizer which is activated by light are main components of PDT. Two types of reactions including Type I reactions which result in formation of free radicals and type II which lead to formation of singlet oxygen cause cytotoxic effects. Because of local property of this treatment and multiple interactions with different cellular organelles and cellular membrane, it seems that development of resistance to PDT could be prevented that is a promising properties compared with systemic antifungal drugs [7–10]. So great amount of interest has been dedicated to utilize Photodynamic therapy (PDT) in the treatment of fungal and bacterial infections and many in vitro and in vivo studies have been published [11–16].

Furthermore, increasing efficacy of PDT is a developing area of interest. In this respect, different protocols including different light source, irradiation time, energy density and especially photosensitizing agents are applying to promote eradication of infections [14–16].

Phenothiazinium derivatives including Methylene Blue and Toluidine Blue have been conventionally used in Photodynamic therapy and showed good results in photoelimination of bacteria and fungi *in vitro* and *in vivo* [17,18].

Indocyanine green (ICG) is an anionic water-soluble tricarboxyanine dye [19]. Activation of the dye at the wavelength of 810 nm resulted in production of singlet oxygen which is one of the main mechanisms of cytotoxic killing. Moreover, photothermal effect has been discussed as a possible mechanism [20–23]. New methylene blue (NMB) N-zinc-chloride-double is a new generation of methylene blue (MB) which with more lipophilic nature [17]. To our knowledge, only limited number of studies investigated application and comparison of both leading dyes and also their lethal effects on *T. rubrum* [24,25]. Rodrigues et al. observed significant photo-inactivation of *T. rubrum* (4-log reduction) after PDT with LED at wavelength of 631 nm, 10 μ M NMB and energy density of 20 J/cm² [24]. Fekrazad et al., assessed and compared the efficacy of NMB and ICG to treat *Candida albicans* fungi and found that aPDT with either EmunDo[®] or new methylene blue (NMB) considerably diminished the viability of inoculated *C. albicans* ($P < 0.001$) by log reduction of 1.9 and 3.37, respectively. Very recently, same authors, investigated the antifungal effect of Citrus aurantifolia essential oil and ICG and reported 75 percent reduction of *T. rubrum* after PDT with ICG at wavelength of 810 ± 10 nm and energy density of 55 J/cm² [25].

The purpose of this study was to compare the effectiveness of photoinactivation of *T. rubrum*, using New methylene blue and Indocyanine green (EmunDo[®]) as two relatively new photosensitizers.

2. Methods and materials

2.1. Microorganism preparation

Trichophyton rubrum reference strain (PTCC 5143), was provided as suspension in sterile phosphate-buffered saline (PBS)

with final concentration of 10⁶ colony forming unit per milliliter (CFU ml⁻¹) (Courtesy of Pasture Institute, Tehran, Iran). Preparation of Sabouraud dextrose agar (SDA, Quelab, Montreal, Canada) medium was performed according to manufacturer's recommendation. 67 g of dehydrated media in 1000 ml of purified filtered water was suspended and was heated with frequent agitation and boil for one minute, Sterilized at 118 °C for 15 min, Cooled to 45–50 °C and Mixed gently and dispensed into sterile Petri dishes or sterile culture tubes. Prior to inoculate, the prepared media brought to room temperature.

The final colony count was reevaluated by sub-culturing on Sabouraud dextrose agar (SDA) in addition to microscopic count.

2.2. Photosensitizer

Stock solution of New methylene blue N zinc chloride double salt (NMBN, Sigma–Aldrich, Germany) (Fig. 1) was prepared by dissolution in phosphate buffered saline (PBS) at 10 μ M concentration. It was stored at 4 °C in the dark till the experiments.

EmunDo[®] solution (A.R.C laser GmbH, Nurnberg, Germany) was prepared according to the manufacturer instruction. The package of EmunDo[®] die which contained prefabricated sterile water vials which was used to prepare 1 mg/gr EmunDo[®] solution.

2.3. Laser sources

InGaAlP red light laser (MUSTANG, Russia) was used as the red laser source. Samples were irradiated with wavelength of 632 nm for 250 s, output power of 10 mW, spot size of 0.8 cm and resultant energy density of 5 J/cm² and.

Photoactivation of EmunDo[®] was carried out by an infra-red diode laser at the wavelength of 810 nm (A.R.C laser GmbH, Nurnberg, Germany) with power output capacity of 300 mW, spot size of 4.5 mm. The samples were irradiated in continuous mode for 30 s with resultant energy density of 55 J/cm². Three parts of each well surface was irradiated by examiner to make sure that the whole surface area of each well was covered by laser beam

2.4. aPDT with NMB and EmunDo[®]

Experiments were performed in 12 predefined wells of the 96-well flat bottomed micro titter plates. The 100 μ l of EmunDo[®] or NMB and 100 μ l of fungal suspension or distilled water was added to predefined wells according to the scheduled groups (Table 1) and was shaken to obtain homogeneous mixture. Before laser irradiation, the mixture of NMB-fungal suspension and EmunDo[®] – fungal suspensions were held in the dark at 28 °C for 30 min and 5 min, respectively, according to manufacturer's recommendation. This time was considered to ensure satisfactory absorption of dyes into *T. rubrum* fungal cells.

Final concentrations of fungal suspension, NMBN and EmunDo[®] were 0.5×10^5 , 10 μ M and 500 μ gr per milliliter, respectively.

In order to avoid the diffusion of laser beam from a well surface to adjacent one, only 12 wells of 96-well flat bottomed micro titter plates were selected for irradiation. So horizontal distance between two adjacent wells was adequate enough in all procedures. For adjustment of vertical distance between laser device and well surface special holders were used in the procedure. After irradiation, suspension were removed and serially diluted in PBS to obtain 10^{-2} – 10^{-4} times of original concentration and finally 10³ μ liter was added to petri dishes containing sabouraud dextrose agar (SDA) in 4 replicates and incubated at 37 °C for 7 days and then the colonies were counted by a blind examiner based on colony forming unit per milliliter.

Continuous data are expressed as mean (standard deviation). Data were analyzed using one-way analysis of variance (ANOVA)

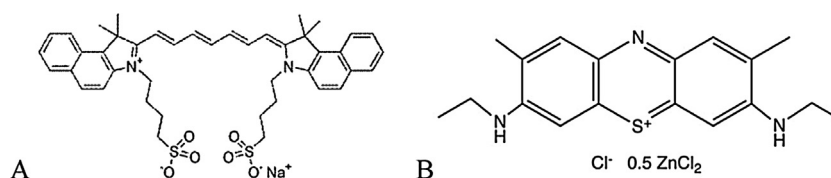


Fig. 1. Chemical structure of Indocyanine green (A) and New methylene blue (B).

Table 1

Description of experimental and control groups. I: ICG group; N: NMB group; RL: red laser; IRL: infra-red laser, RLN: red laser + NMB; IRLI: infra-red laser + ICG; control: only fungal suspension; I and N: dye-only groups; RL and IRL: laser-only groups; RLN and IRLI: aPDT groups.

Experiments			
100 μ liter of fungal suspension	100 μ liter of ICG +	No laser irradiation	1.I
100 μ liter of fungal suspension	100 μ liter of NMB+	No laser irradiation	2.N
100 μ liter of fungal suspension	100 μ liter of distilled water +	Red laser irradiation +	3.RL
100 μ liter of fungal suspension	100 μ liter of distilled water +	Infrared laser + irradiation	4.IRL
100 μ liter of fungal suspension	100 μ liter of NMB +	Red laser irradiation +	5.RLN
100 μ liter of fungal suspension	100 μ liter of + ICG	Infrared laser + irradiation	6.IRLI
100 μ liter of fungal suspension	100 μ liter of distilled water +	No laser irradiation	7.Control ©

and Games–Howell test for further multiple comparisons due to significant Levene test. A two- tail P value less than 0.05 was considered as statistical significance cut point.

3. Results

3.1. General finding

The colony count of the some experimental groups (IRLE, E, RLN) were considerably lower than the control group which received neither laser nor dye ($P < 0.05$).

A marginal reduction of colony count was noticed with aPDT with EmunDo[®] ($P = 0.07$) and aPDT with NMB achieved more reduction ($P = 0.003$). Almost same pattern was observed when log reduction was taken into account for aPDT with EmunDo[®] ($P = 0.2$) and aPDT with NMB ($P = 0.02$) with respective reduction of 0.64log and 0.4log compared to the control group (Table 2, Supplementary material).

3.2. Antimicrobial photodynamic therapy

No statistically significant difference was observed between two groups of aPDT (IRLE: EmunDo[®], 810 nm and RNL: NMB, 630 nm) in reduction of CFU counts ($P = 0.73$).

3.3. Laser only groups

No significant difference was found between two light groups ($P = 0.79$) (IRL: 810 nm vs. RL: 630 nm), though irradiation of *T. rubrum* with only red laser reduced viability of cells compared to control group ($P = 0.04$).

3.4. Dye only groups

The difference between potency of EmunDo[®] alone and new methylene blue alone did not reached statistical significance ($P = 0.14$). A remarkable reduction was only observed in Emudno only group when compared to the control group ($P = 0.04$).

4. Discussion

In the present study, we assessed the efficacy of the photo-inactivation of *T. rubrum* by two photosensitizing agents, new methylene blue (NMB) and Indocyanine green (EmunDo[®]). A significant reduction of *T. rubrum* cells by either of ICG- or NMB-derived

antimicrobial photodynamic therapy was achieved compared to the control group ($P < 0.05$). Data are scarce evaluating the efficacy of aPDT with NMB and ICG to eradicate *T. rubrum* fungal cells.

aPDT with NMB reduced the *T. rubrum* cells by almost 0.4 log₁₀ that was similar to reduction rate by red laser alone photo-inactivation. Previously, Rodrigues et al., demonstrated 1 log₁₀ reduction of *T. rubrum* cells when they applied energy density of 5 J/cm² and 10 μ M concentration of NMB. While energy density, wavelength and dye concentration were similar in present and mentioned study, final concentration of *T. rubrum* cells was 2×10^6 cells/ml which was 40 fold higher than this work (0.5×10^5). High availability of NMB per cell in this study may lead to saturation of NMB targets in *T. rubrum* cells and according to Jackson et al., high amount of unbound dye can absorb photons away from NMB-cell complex [26]. This is the probable reason that why in present study, reduction of cell viability was even less than the study by Rodrigues. In the future, it is necessary to determine the optimum ratio of cell density to dye in which more effective photodynamic inactivation could be happened. Further- more, determination of Minimum inhibitory concentration for both of the dyes will definitively help to this purpose. Same authors found that treatment with light exposure (LED 632 nm) only or NMB in the absence of light did not inhibit growth of *T. rubrum* which is not in agreement with this study [24].

In this study cell toxicity of NMB alone was not remarkable (0.14 log₁₀), yet red laser alone eliminated higher portion of cells. While no other study was found evaluating PDT with NMB on *T. rubrum*, superiority of red laser to infra-red laser source to eliminate fungal cells may be related to the internal structure organelles of fungal cells. Fungal cells possess internal pigments such melanin with peak absorbance at 639 nm and red pigments with peak absorbance at 532 nm [27,15]. Transmission of laser energy to these pigments may be followed by increases temperature and damage to other cell vital structures. Peak absorbance of existing pigments match better with red laser source rather than infra-red laser source [27,15]. Another possible explanation for different inhibitory effects of two lasers may be correlated to Energy density and wavelength of each laser used in the study. Application of red laser ($\lambda = 630$ nm, Energy Density of 5 J/cm²) may increase inhibitory effect of laser on *T. rubrum* via changes in internal organelles, enzymes and interfering with respiratory chain and nucleic acids. So this inhibitory effect may be more pronounced with mentioned parameters of red laser ($\lambda = 630$ nm, Energy Density of 5 J/cm²) compared with infra-red laser ($\lambda = 810$ nm, Energy Density 55 J/cm²). Similar pattern of inhibitory or stimulatory effect was observed by Nussbaum et al.,

Table 2

Mean + SD of CFU/ml and its reduction.

	IRLI	IRL	I	CO	RLN	N	RL
Count (CFU/ml, E ^{5*})	1.3(0.87) ^{d,f}	3.3(1.15)	1.6(0.65) ^{d,f}	5 ^{a,c,e,g}	2.13(0.43) ^d	3.68(0.1) ^{a,c,d}	2.25(0.83) ^d
Reduction (%)	74(17)	33(23)	51(15)	0.01	58(9)	27(21)	55(12)

I and N: dye-only groups; RL and IRL: laser-only groups; RLN and IRLI: aPDT groups

Superscripts indicate the presence of statistically significant differences (Games-Howell: $p < 0.05$).*E5 indicates that the data should be multiplied by 10⁵.^bIRL.^cRL.^aIRLI.^cI.^dCO (control).^eRLN.^fN.

when they administered same parameters for E.coli, inhibitory effect of red laser and stimulatory effect of infra-red laser was predominant [28]. On the other hand, We did not find significant difference between red laser(RL) and RLN (red laser and NMB) on *T. rubrum* cells. One probable explanation is related to light dose of 5 j/cm² applied in the study. It seems that the mentioned dose did not provoke the NMB dye effectively and photodynamic reaction was deficiently occurred. More photodynamic inactivation would occur in light dose of >5 j/cm² as reported by rodriguez et al. [24].

The photoactivated effect of Indocyanine green (EminDo[®]) has been also evaluated in this study. The dye was previously approved by FDA and widely applied in medical diagnosis [19]. About 98 percent of the dye binds to the plasma proteins. Unbound form of ICG is rapidly excreted to bile duct [29]. High affinity to absorb the light in near infra-red region ($\lambda = 810$ nm) enhances its penetration depth compared to other photosensitizers [29]. So it seems that the dye has good properties to act as a photosensitizer [19,30,31]. Production of singlet oxygen is a crucial component in cytotoxic action of the dye following photodynamic therapy. Furthermore photothermal effect has been implicated to play adjunctive role in its antimicrobial action [32,33].

A better result was found with ICG-derived photoinactivation (0.64 log₁₀, %74 CFU/ml reduction), which was relatively similar to previous work by Fekrazad et al. [25]. The efficacy of infra-red laser alone was not significant (0.19 log₁₀ reduction), however, better outcomes was achieved with ICG alone dark toxicity (0.51 log₁₀), which are comparable with Fekrazad et al., findings [25]. On the contrary, ICG exhibited higher fungicidal effect compared to NBM. In this study, we have used ICG at the concentration of 500 µg/ml. This concentration was opted due to two reasons: First, the fact of negative charge of both ICG and *T. rubrum* cells. The second reason was that a minimum concentration of 200 µg/ml was suggested to eliminate gram negative bacteria [34]. Fungal cells are much larger than bacterial cells and have cell walls as less permeable barrier to be penetrated by external dye molecules [34,35]. Hence we conducted our research with the mentioned concentration of ICG [25]. Despite the positive charge of NMB, this phenothiazine photosensitizer was not fungicidal when applied alone. Studying the pattern of staining of various fungal cells organelles and cell walls by means of fluoroscopic evaluation may shed light on this controversial issue. Probably other yet not known mechanism may be attributing to explain to this matter.

Another possible mechanism for better result of photoinactivation by ICG compared with NMB was mentioned by Eagle et al. They mentioned that when ICG is irradiated by infrared light (diode laser, 810 nm, radiant exposure 20 j/cm²), not only does it produce singlet oxygen, but also singlet oxygen oxidizes the ICG molecule itself, resulting decomposition of ICG. So the decomposed products inside or near cells can also damage cells and decrease their viability. So this mechanism is able to some extent reinforce dark toxicity of ICG mediated photodynamic therapy [23].

Considering that dark toxicity of both lead dyes (especially EminDo[®]) was considerable in our study, The possible effects of both dyes on natural host cells are important to note, especially at high concentrations and the precise minimum inhibitory effect or optimal therapeutic dose must be determined while considering safety precautions.

One possible solution to avoid the side effect of photosensitizing agents is application of novel laser treatment modalities. For example, combination of ultrasound and ICG mediated photodynamic therapy has been shown better results in eradication of tumor cells [36].

It is concluded that reduction of *T. rubrum* cells by either of ICG- or NMB-mediated antimicrobial photodynamic therapy was significant compared to the control group ($P < 0.05$).

Dark toxicity of ICG and effect of 630 nm red laser on viability of *T. rubrum* seems to reach significant level compared to control group. So it is required more evidence-based data to determine the absorbance and scattering characteristics of *T. rubrum* and determine precise light parameters and photosensitizer properties.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pdpdt.2016.10.013>.

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